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Short communication

Validated HPLC analytical method with programmed wavelength UV detection for simultaneous determination of DRF-4367 and Phenol red in rat in situ intestinal perfusion study☆

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Abstract

A simple, precise and accurate isocratic reverse-phase liquid chromatography method with programmed wavelength detection has been validated to quantify DRF-4367 and Phenol red, simultaneously for application in rat in situ single pass intestinal perfusion study to assess intestinal permeability of DRF-4367, a novel cox-2 inhibitor. The method was validated on RP C-18 analytical column. Mobile phase consisted of sodium dihydrogen orthophosphate (pH 3.2, 0.01 M)–acetonitrile–methanol (30:50:20, v/v/v). The developed method has a short run time of 12 min with a flow rate of 1.0 ml/min. The injector volume was set to 20 μl and acquisition was carried out using a PDA detector while processing was done by timed wavelength extraction. The percentage R.S.D. and recovery in all studies indicated that the method was suitable for the intended purpose. The validated method was found to be linear and precise in the working range. Suitability of storage at cold temperature was ensured along with complete sample recovery.

Keywords: Reverse-phase liquid chromatography; Intestinal permeability; Validation

1. Introduction

An estimated 40% of new molecular entities fail to be new drugs because of poor biopharmaceutical properties, namely solubility and permeability [1]. Permeability is an important factor, which governs absorption of orally administered drugs. Solubility is easily quantifiable in vitro and can be manipulated by formulation strategies, while permeability is more complex to be altered for improved performance in human beings. Hence, screening of drug candidates for permeability properties is imperative to select right candidate for development to prevent late surprises [2]. Of all permeability screening methods, in situ intestinal perfusion study offers a simple and relevant method of permeability assessment and correlates very much with the true absorption properties in human beings [3]. The method primarily involves perfusing a short intestinal segment (jejunum) with a known concentration of the test substance in physiological buffer; the outlet concentration of the test substance is estimated from the perfusate at the distal end [4]. The difference of concentration in inlet to outlet is translated to effective permeability values. The concentration changes could also be affected by the water content of the intestine, calling for use of volumetric markers that have zero permeability like Phenol red [5]. The concentrations of the test substance and zero permeability marker used in the study are generally of low concentrations

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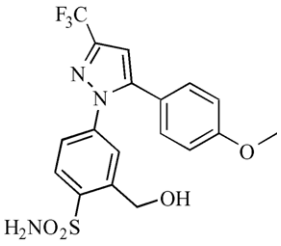

Fig. 1. Molecular structure of DRF-4367, a novel cox-2 inhibitor.

in the order of 5–50 μg/ml (10–100 μM), which calls for a reliable and reproducible analytical method. As can be anticipated, the role played by the analytical method, is of major significance and the process of method development and validation has direct impact on quality of data. A validated analytical method shown to have the capacity to keep the fundamental characteristics deemed essential for successful study.

In this study, we report a validated HPLC method for simultaneous determination of DRF-4367 and Phenol red in intestinal perfusate. DRF-4367, a novel cox-2 inhibitor, is a diaryl pyrazole derivative (Fig. 1), shown to have a low bioavailability of 20% at dose of 100 mg/kg in rats [6].

Permeability properties of DRF-4367 by rat in situ intestinal single pass perfusion study were evaluated as possible limiting factor in poor oral bioavailability. DRF-4367 has low solubility in the pH range of 2–10, posing the challenge of formulating the solution in physiological buffers for perfusion [7]. As a result a 0.16% hydroxy propyl-β-cyclodextrin (HPβCD) solution is used. Although the use of *N*-methyl pyrrolidone has been practiced for insoluble drug substances, our approach to use HPβCD was mandatory because this drug was formulated in HPβCD vehicle to enhance solubility and the use of HPβCD at such levels (0.16%) is less likely to impart permeability changes [8]. The method was validated with respect to linearity, precision, storage-stability, system suitability and recovery in presence of perfusion matrix. The parameters and validation protocol adopted in this work is not necessarily in accordance with ICH or US FDA guidelines but to suit the intended scientific investigation.

2. Materials and methods

2.1. Materials

DRF-4367 was synthesized by the discovery chemistry division of Dr. Reddy's Laboratories Limited, Hyderabad, India. Phenol red, water soluble pH indicator (Qualigens), methanol (Merck, HPLC grade, Lichrosolv), acetonitrile (Merck, HPLC grade, Lichrosolv), sodium dihydrogen orthophosphate (ExcelaR grade, Qualigens, Mumbai, India), orthophosphoric acid (Merck, G.R grade, minimum purity 88%) and HPβCD, pharma grade (Cavasol W7 pharma, Wacker chemical, Germany).

2.2. Equipment

Waters alliance 2695 separations module appended with 2996 PDA detection system, Waters Empower software, Version 5.0, Thermo Orion Model 420A+ pH meter.

2.3. Chromatographic conditions

Inertsil ODS-3 RP C-18 (250 mm × 4.6 mm, 5 μm) was used for the validation studies. The column temperature was set at 35 °C. Freshly prepared mobile phase was filled in the reservoirs and run in an isocratic mode. The mobile phase was run at a flow rate of 1.0 ml/min with a run time of 12 min and an injection volume of 20 μl. Acquisition was carried out with PDA detector in the wave length range of 210–600 nm; extraction of chromatograms was carried out by timed wavelength at 421 and 245 nm, λ_{max} of Phenol red and DRF-4367, respectively.

2.4. Preparation of mobile phase

The mobile phase used for the chromatographic separation comprised of aqueous buffer, acetonitrile and methanol in the ratio of 30:50:20 (v/v/v). Aqueous buffer solution contains sodium dihydrogen orthophosphate (pH 3.2, 0.01 M) and its pH is adjusted with 10% orthophosphoric acid and filtered through a 0.22 μm PVDF membrane using a Millipore vacuum filtration system and degassed by sonication. Organic solvents filtered through 0.22 μm (PVDF) filters were also used after degassing.

2.5. Sample preparation

Stock solution of DRF-4367 and Phenol red was prepared by dissolving accurately about 20 mg of DRF-4367, Phenol red and combination in methanol. The stock solutions were further diluted serially with mobile phase to get the required concentrations. All standards were freshly prepared for every study.

For recovery studies, formulation of DRF-4367 and Phenol red in 0.16% HPβCD solution was prepared at a concentration of 1 mg/ml each in combination and aliquots of the formulation was spiked into the blank rat intestinal perfusate. Vortexing was done for 5 min. The samples were diluted with mobile phase in the ratio of 1:1 and centrifuged at 10,000 rpm for 15 min. The clear supernatant was filtered through 0.22 μm PVDF membrane filter into HPLC vials and injected for analysis. All the validation studies were carried out with respect to DRF-4367, Phenol red individually and in combination.

2.6. Linearity

Five standard concentrations of 10, 20, 50, 100 and 200 μg/ml covering the entire range of expected concentration in perfusate were prepared by serially diluting a 200 μg/ml solution of both. Accurately about 20 mg of DRF-4367 and Phenol red was weighed and was dissolved in 100 ml of mobile phase. Combination of DRF-4367 and Phenol red was made by dissolving about 20 mg of both DRF-4367 and Phenol red in 100 ml of mobile phase. All the solutions were filtered through 0.22 μm PVDF filters into HPLC vials. All the injections were carried out in triplicate.

2.7. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision was considered as system, method precision and repeatability assay.

2.8. System precision and method precision

System precision was determined by analyzing six replicates of 100 μg/ml of DRF-4367, Phenol red and their combination from the same homogenous solutions. The % R.S.D. was calculated for the replicates as a measure of system precision. For method precision five independent solutions, 100 μg/ml each of DRF-4367, Phenol red and their combination was injected; the variation in the results was estimated in terms of % R.S.D. of the determinations.

2.9. Intermediate precision

Intermediate precision was assessed at four concentrations (10, 25, 50 and 100 μg/ml) covering specified range. All the injections were carried out in triplicates. Intermediate precision was considered at two levels, intra day and inter day.

Intra day precision data were obtained by analyzing three sets of freshly prepared standards in one laboratory in one day. The chromatographic peak areas were compared and the % R.S.D. between measurements was estimated as a measure of intra day precision.

Inter day precision was measured as % R.S.D. between analysis of standards described in method procedure on three consecutive days in the same laboratory; all the standards and mobile phase were freshly prepared for the assay.

2.10. Bench top and freeze thaw storage-stability suitability studies

The suitability of sample storage at lower temperature and holding on bench top during the perfusion study and prior to processing was evaluated. The bench top stability study was carried out by holding one set of samples on the bench top experimental conditions for 24 h. Another set of samples was subjected to three cycles of freezing at −4 °C and thawing to room temperature (22 ± 2 °C) in cycle duration of 24 h. Both the studies were carried out at three concentration levels of 10, 25 and 50 μg/ml in triplicates. Peak area of the analyte in test solutions and freshly prepared standard solution were compared in terms of % R.S.D. of test samples against mean with fresh samples.

2.11. Recovery

Recovery is the ratio of amount of compound of interest analyzed to theoretical amount present in the medium. Rat jejunal segment was perfused with freshly prepared Tyrode's buffer and collected at the outlet. One milligram per millilitre formulation of DRF-4367 and Phenol red prepared in 0.16% HPβCD solution was spiked in aliquots of intestinal blank perfusate to get a concentration of 10, 25 and 50 μg/ml. The samples were prepared in triplicates. The resultant solutions were vortexed for 3 min and diluted 1:1 with mobile phase and centrifuged at 10,000 rpm for 10 min after thorough vortexing. The clear supernatant was filtered through 0.22 μm PVDF filter into HPLC vials and analyzed in triplicate injections. Another set of neat standards in mobile phase was prepared and injected in triplicates. The recovery was determined by comparing the peak areas of the spiked rat perfusion extracts and neat standards prepared in mobile phase.

3. Results and discussion

Initial method development trials to optimize mobile phase for better resolution of Phenol red and DRF-4367 were done with aqueous buffer (pH 3.2) and acetonitrile. Zero to twenty percent of methanol was tried by incorporation in mobile phase to enhance peak resolution and symmetry. It was found that the peak symmetry and maximum separation could only be achieved at the mobile phase composition of sodium dihydrogen phosphate (pH 3.2, 0.01 M), acetonitrile and methanol in the ratio of 30:50:20 (v/v/v).

Flow rates of 0.5, 1.0 and 1.5 ml/min indicated that at 1 ml/min, the peak symmetry was better and has shown good resolution of Phenol red and DRF-4367 with RT values of 4.6 and 5.9 min, respectively.

λ_{max} of Phenol red and DRF-4367 were found to be 421 and 245 nm, respectively. In order to get a maximum peak response, the extraction of the PDA acquisition was carried out by programmed/timed wave length corresponding to the λ_{max} at the respective RT, as shown in Fig. 2.

3.1. Suitability of method

Chromatographic parameters such as resolution, selectivity and peak asymmetry were satisfactory for both the compounds given in Table 1. The calculated resolution between DRF-4367 and Phenol red was not less than 2.90

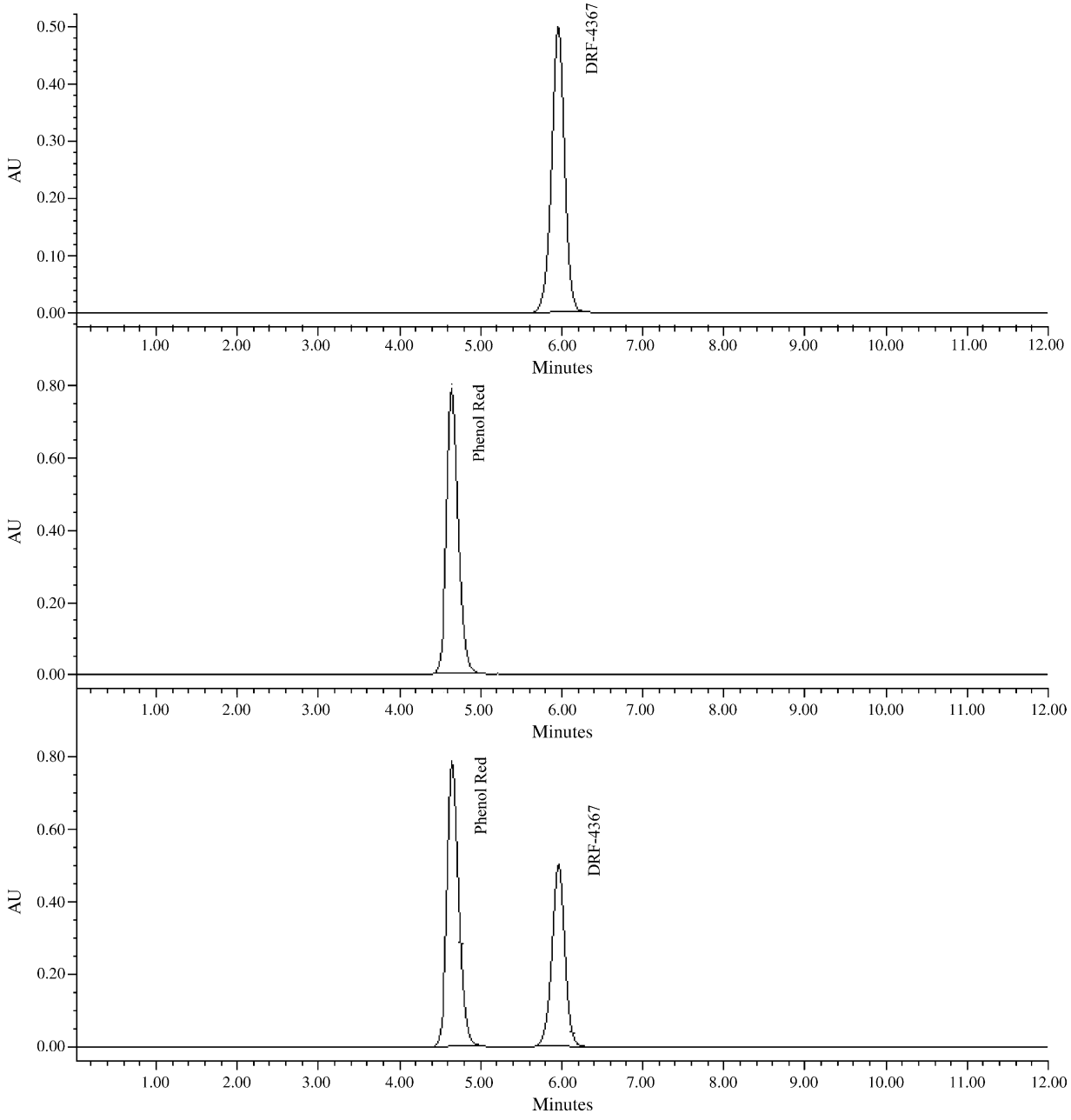

Fig. 2. Representative chromatogram of DRF-4367 and Phenol red individually and in combination.

and selectivity was above 1.32. K' values were found to be greater than 3 for both Phenol red and DRF-4367, respectively. Number of theoretical plates (per meter of the column) and tailing factor were observed to be satisfactory.

3.2. Linearity

Linearity study verifies that the sample solutions are in the concentration range where the analyte response is

Table 1
System suitability parameters of DRF-4367 and Phenol red, average of nine readings

Sample name		*RT*	USP tailing	USP plate count	Resolution	K'
Individual	DRF-4367	5.510 ± 0.015	1.150 ± 1.418	56050 ± 6.020	Not applicable	4.510 ± 0.015
	Phenol red	4.228 ± 0.068	1.235 ± 1.965	8432 ± 2.520		3.228 ± 0.091
Combination	DRF-4367	5.510 ± 0.010	1.030 ± 1.312	55088 ± 5.349	9.157 ± 1.359	4.510 ± 0.014
	Phenol red	4.247 ± 0.095	1.232 ± 1.537	8474 ± 2.901		3.247 ± 0.123

Table 2
Linearity parameters of the HPLC analytical method of DRF-4367 and Phenol red

Parameter	DRF-4367	Phenol red	Combination	
			DRF-4367	Phenol red
Range (μg/ml)	10–200	10–200	10–200	10–200
Intercept	18907	286457	226657	651752
Slope	54296	68694	59817	56918
r^2	0.99998	0.9994	0.9982	0.9848

linearly proportional to the concentration. The linearity study was carried out in range of 10–200 μg/ml covering the possible concentration range in perfusate analysis deduced from initial trial experiments. Table 2 describes regression statistics obtained for the various analytical tests. The linearity of the method was observed in expected concentration range, demonstrating suitability for the analysis. The goodness of the fit (r^2) was found to be 0.99998 for DRF-4367, 0.9994 for Phenol red individually and 0.9982 for DRF-4367, 0.9848 for Phenol red in combination.

3.3. Precision

3.3.1. System and method precision

System and method precision were evaluated prior to conducting validation studies. Injecting multiple samples from the same homogenous solution indicates the performance of the system excluding possibility of all other variations. The % R.S.D. of the measurements was in all cases found to be less than 0.3. Method precision measures the closeness of analytical results when six separately prepared standards are injected. The % R.S.D. was found to be less than 1.5 in all cases. The detailed results are given in Table 3. The values indicate that the method is precise.

3.3.2. Inter day and intra day precision

The degree of reproducibility of the method when samples are analyzed over period of time is measured in terms of intra day and inter day precision. Chromatographic peak areas of the analysis were used as comparative parameter. The concentrations were deduced from the linearity plots. The highest value of % R.S.D. analysis was found to be 1.64 for selected concentrations for inter day measurements, while it was found to be 1.89 for intra day measurements. The values indicated that the method is accurate and precise. The results are given in Table 3.

3.4. Bench top holding and freeze thaw storage suitability

The suitability of holding samples on the bench top during the study, storage at frozen conditions and thawing prior to processing was evaluated with an aim to address possible unanticipated issues necessitating storage or holding of the samples after experiment prior to sample processing.

Samples of DRF-4367 and Phenol red were divided into three lots, one set injected for analysis as 0 h samples, second held on bench top at experimental conditions for 24 h and third subjected to three cycles of freezing and thawing. All the studies were carried out at three concentration levels, high, intermediate and low. The results analyzed as % R.S.D. of the test determinations against mean with 0 h are given in Table 3. The highest value of % R.S.D. was found to be 1.7 in case of bench top and 2.36 for freeze thaw studies and expresses suitability of storage. The detailed results at every concentration level are given in Table 3.

3.5. Recovery

Recovery is a measure of the extent of analyte recovered from the test matrix so as to minimize the errors of loss in extraction, amounting to errors in the experiment. Recovery studies were performed by spiking a known concentration of analyte in the matrix and the extent recovered in analysis is translated to percent recovered. Recovery studies with DRF-4367 and Phenol red in rat intestinal perfusion matrix indicate the steps involved from point of sample collection to analysis do not deviate much from the permitted limits. The highest value of % R.S.D. was found to be 1.84 in all cases. Table 3 gives the detailed results of recovery.

4. Applicability

Validated analytical method was applied for rat in situ intestinal perfusion study to assess intestinal permeability of DRF-4367. The study was carried out in male Sprague–Dawley rats ($n = 6$) at 50 μg/ml of DRF-4367 (molecular wt = 441.42 and 50 μg/ml of Phenol red. The study protocol was approved by in-house animal ethical committee. Both DRF-4367 and Phenol red were formulated in 0.16% HPβCD. Inlet and outlet samples were processed and analyzed for DRF-4367 and Phenol red. The concentration of DRF-4367 and Phenol red in the processed outlet perfusate was in the range 15–25 μg/ml. The difference of the concentration of Phenol red was attributed to the flux of water and corresponding concentration correction of DRF-4367 was carried out. The permeability coefficient of DRF-4367 is calculated using Eq. (1).

$$P = \frac{q_i}{LE_p} \ln\left(\frac{C_i C_{Mo}}{C_o C_{Mi}}\right)\left(\frac{(C_{Mi}/C_{Mo}) - 1}{\ln(C_{Mi}/C_{Mo})}\right) \quad (1)$$

where C_i and C_o are inlet and outlet concentrations of DRF-4367 while C_{Mi} and C_{Mo} are inlet and outlet concentrations of Phenol red. q_i, L and E_p represent volumetric flow rate of perfusate, length and perimeter of the intestinal segment perfused, respectively.

The term $\left(\frac{(C_{Mi}/C_{Mo})-1}{\ln(C_{Mi}/C_{Mo})}\right)$ corresponds to the volumetric change of the water and is always around 1 ranging from 0.924 to 1.163. It is here that the concentration of the

Table 3
Experimental value of mean concentration S.D. and % R.S.D. given for validation parameters of DRf-4367 and Phenol red

Study	Sample size	Concentration (μg/ml)	Individual				Combination			
			Mean (DRF-4367 ± S.D.)	% R.S.D.	Mean (Phenol red ± S.D.)	% R.S.D.	Mean(DRF 4367 ± S.D.)	% R.S.D.	Mean(Phenol red ± S.D..)	% R.S.D.
System precision	5	100	100.37 ± 1003.7	0.10	100.75 ± 2418.0	0.24	98.69 ± 2862.0	0.29	100.46 ± 2310.6	0.23
Method precision	6	100	100.68 ± 12081.6	1.20	99.35 ± 8643.5	0.87	100.66 ± 9562.7	0.95	100.11 ± 14015.4	1.40
Inter day	3	10	10.15 ± 284.2	0.28	9.82 ± 1080.2	1.10	9.89 ± 563.7	0.57	10.01 ± 210.2	0.21
		25	25.00 ± 475.0	0.19	24.90 ± 3461.1	1.39	24.61 ± 2756.3	1.12	24.73 ± 2769.8	1.12
		50	49.69 ± 8149.2	1.64	49.86 ± 648.2	0.13	50.75 ± 4009.3	0.79	49.88 ± 6434.5	1.29
		100	99.51 ± 6070.1	0.61	99.52 ± 4179.8	0.42	99.67 ± 3687.8	0.37	100.24 ± 5513.2	0.55
Intra day	3	10	9.84 ± 196.8	0.20	9.75 ± 487.5	0.50	9.93 ± 665.3	0.67	9.85 ± 768.3	0.78
		25	25.18 ± 3021.6	1.20	25.23 ± 756.9	0.30	25.07 ± 1228.4	0.49	25.50 ± 1683.0	0.66
		50	50.36 ± 9518.0	1.89	50.51 ± 2626.5	0.52	50.50 ± 909.0	0.18	50.01 ± 3100.6	0.62
		100	99.80 ± 12974.0	1.30	99.72 ± 3789.4	0.38	99.65 ± 1694.1	0.17	99.90 ± 6393.6	0.64
Recovery	3	10	9.89 ± 1523.0	1.54	9.92 ± 1349.1	1.36	9.84 ± 1535.0	1.56	9.94 ± 1828.9	1.84
		25	24.67 ± 2343.7	0.95	25.42 ± 1143.9	0.45	25.14 ± 2815.7	1.12	24.85 ± 2609.3	1.05
		50	50.04 ± 2201.8	0.44	50.14 ± 1604.4	0.32	50.12 ± 4861.6	0.97	50.33 ± 4429.0	0.88
Bench top	3	10	10.01 ± 690.7	0.69	10.17 ± 203.4	0.20	10.10 ± 1717.0	1.70	10.04 ± 1656.6	1.65
		25	25.02 ± 525.4	0.21	24.80 ± 1041.6	0.42	24.39 ± 3121.9	1.28	24.63 ± 1527.1	0.62
		50	50.35 ± 5337.1	1.06	50.13 ± 551.4	0.11	49.93 ± 2895.9	0.58	100.46 ± 2310.6	0.46
Freeze thaw	3	10	10.17 ± 996.7	0.98	10.23 ± 797.9	0.78	10.27 ± 1006.5	0.98	100.11 ± 14015.4	0.78
		25	25.67 ± 2746.7	1.07	25.55 ± 2631.7	1.03	25.88 ± 2769.2	1.07	10.01 ± 210.2	1.03
		50	50.29 ± 11868.4	2.36	49.83 ± 2142.7	0.43	50.15 ± 11835.4	2.36	24.73 ± 2769.8	0.47

Phenol red in the outlet concentration affects the calculation, which could be incorporated by a poor analytical method. The permeability co-efficient of DRF-4367 was found to be 2.576×10^{-5} cm/s.

5. Conclusion

A simple, rapid, precise, accurate and reproducible HPLC method using C18 column was validated with programmed wavelength UV detection for simultaneous determination of DRF-4367 in rat in situ single pass intestinal perfusion studies. The validated method was successfully applied to the rat in situ single pass intestinal perfusion studies of DRF-4367 and was found to be suitable for the intended purpose incorporating a high degree of confidence in the experimental data preventing possibility of errors from analysis.

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